Early Activation of Human Vγ9Vδ2 T Cell Broad Cytotoxicity and TNF Production by Nonpeptidic Mycobacterial Ligands[1]

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Human Vγ9Vδ2 T cells were shown recently to respond to nonpeptidic phosphorylated molecules of mycobacterial origin (previously referred to as TUBag). To investigate the early events of Vγ9Vδ2 T cell activation, we have analyzed induction of cytotoxicity and TNF production of T cell clones by these molecules. We showed that within minutes after exposure, TUBag induced cytotoxicity of Vγ9Vδ2 CTL (but not of CTL expressing other TCR Vγ/Vδ or Vα/Vβ regions) against a broad set of target cells, including effector cells themselves. Induction of Vγ9Vδ2 cytotoxicity by TUBag was blocked by anti-TCR mAbs and was abrogated after dephosphorylation of TUBag. Similarly, TUBag, but not dephosphorylated TUBag, induced massive TNF production by Vγ9Vδ2 T cell clones only, which already was significant 20 min after exposure. Of note, only basal amounts of TNF were produced when cells were maintained in suspension in the presence of TUBag, indicating that efficient activation of TNF production induced by these compounds required a cell-to-cell contact. Finally, preincubation experiments allowed us to demonstrate that activation of Vγ9Vδ2 T cells was strictly dependent on the presence of TUBag because preincubation of the targets with TUBag followed by a single wash abrogated the activation. Taken together, these results strongly suggest that activation of Vγ9Vδ2 cells by TUBag occurs after binding of these compounds to (a) yet unidentified, highly conserved, and broadly distributed molecule(s). The results also suggest either that TUBag induces a very rapid and transient expression of a Vγ9Vδ2 TCR ligand or, more likely, that TUBag is a low affinity component of a complex recognized by the Vγ9Vδ2 TCR. *The Journal of Immunology,* 1995, 154: 5986–5994.

A lthough the precise function of γδ T cells yet remains elusive, numerous studies performed during the last five years have evidenced several features distinguishing this subset from αβ T cells. Among these, the tropism of several γδ T cell subsets for epithelia in direct contact with the external environment, their marked reactivity toward conserved mycobacterial and tumoral Ags and their ability to interact with nonprocessed Ags are probably the most salient ones (for review, see Refs. 1, 2).

The prokaryotic and eukaryotic Ags recognized by γδ T cells appear to be extremely heterogeneous. Murine and human γδ T cells can recognize MHC-related molecules (e.g., classical and nonclassical MHC products) (3–7), MHC-unrelated molecules belonging to the Ig gene superfamily (e.g., CD1 (8), CD48 (9), Ig (10)), heat shock proteins (HSP)[3] (11–14) as well as other native glycoproteins (15).

Besides protein Ags, nonpeptidic phosphorylated molecules of mycobacterial origin were shown recently to trigger the proliferation of a major human γδ T cell subset expressing Vγ9 and Vδ2 TCR regions (16–18). However, the precise mode of action of these so-called TUBag (17)

[3] Abbreviations used in this paper: HSP, heat shock proteins; LA, leukoagglutinin; BLCL, B lymphoblastoid cell line; PI, propidium iodide; ME, mycobacterial extract.

has remained unclear. On the basis of blocking experiments using Abs raised against pro- and eukaryotic HSP, it has been suggested previously that bacterial ligands for Vγ9Vδ2 TCR were derived from stress proteins homologous to *Escherichia coli* GroEL HSP (19, 20). Hence, this raised the possibility that TUBag-phosphorylated compounds could be capable of pre-activating Vγ9Vδ2 T cells in a soluble form and independently of the TCR, and that only in a second phase would Vγ9Vδ2 TCR interact specifically with its physiological ligand (HSP or others) on the target cell. To address this point, we analyzed early activation events such as induction of cytotoxicity and TNF production after exposure to TUBag. The data herein rule out activation of Vγ9Vδ2 T cells by soluble TUBag and show, instead, that TUBag must be bound to the target cells on what appear to be conserved and broadly distributed molecules before Vγ9Vδ2 activation. Moreover, although our data do not formally rule out an induction process, they are more compatible with the hypothesis of a direct recognition of these mycobacterial ligands by Vγ9Vδ2 TCR (presumably in association with their putative receptors), which would result in an almost immediate activation of T cell broad killing activity and TNF production.

Materials and Methods

Abs

The following mAbs were used for flow cytometry analyses and functional assays: IMMU49 (Vγ9Vδ2 G115 clonotype-specific) (21), IMMU360 (anti-Vγ9) (21), IMMU389 (anti-Vδ2) (21), IH1 (anti-Vδ1) (I. Houde, M. A. Peyrat and M. Bonneville, unpublished material), PL8 (anti-HLA DR framework), and 2D6 (anti-HLA DP/DR framework, W6/32 (anti-HLA class I framework). PL8, 2D6, and W6/32 mAb were obtained from the VIIth HLA Workshop.

Preparation of mycobacterial extracts, purification of the TUBag mycobacterial compounds, and enzymatic treatments

The purification procedure for the *Mycobacterium tuberculosis*-derived TUBag was performed as described previously (17). In brief, mycobacteria were killed by overnight incubation in agitated chloroform-methanol (v/v) solvent. The chloroform-methanol extract was dried, resuspended in chloroform-water (v/v), and left to decant before collecting the aqueous phase, which was then dried to yield mycobacterial extracts (ME) reconstituted to 100 mg/ml in water as stock solutions stored at −30°C. For separation of TUBag, ME were subjected to DEAE anion exchange chromatography, eluted with 0.2 M salt, separated in isopropanol + 30% water from silicic acid chromatography. The active material was separated in the water eluent from reverse phase open column chromatography and further in ion pair reverse phase-HPLC on C18 columns (Bischoff Chromatography, Leonberg, Germany) before isolation by HPAEC using AS11 columns and dual conductimetry plus UV detection. Each separation step was monitored by detection of the proliferative or cytotoxic responses of a Vγ9Vδ2 clone (G115) as described previously (17). Enzymatic treatments were done on ME or purified TUBag4 using 3 U of calf intestinal alkaline phosphatase (Boehringer, Mannheim Meslan, France) plus 1 U of *Crotalus adamanteus* venom nucleotide pyrophosphatase (Sigma Chemical Co., Saint Quentin, France) incubated overnight at room temperature before assay for activity. When not specified, TUBag or ME were tested at 100 arbitrary stimulating U/ml, which correspond to ~100 ng/ml purified material in the case of TUBag4 (17).

T cell clones

T cell clones were derived from PBL from healthy individuals (22, 23) and from skin-infiltrating lymphocytes from a patient undergoing an acute graft-vs-host disease (24). They were isolated by limiting dilution and cultured as described previously (24). TCR-variable region expression was deduced from flow cytometry analysis using TCR V-specific mAbs and from molecular analysis of TCR gene transcripts (22, 23).

Cytotoxicity assays

Cytolytic activity of T cell effectors was estimated in short-term ^{51}Cr-release assays as described previously (24). CTL lytic potential was estimated by adding purified PHA (leucoagglutinin (LA) (Pharmacia, Uppsala, Sweden) at 1 μg/ml during the assay. In a set of preliminary experiments, we observed that efficient activation of CTL lytic machinery by TUBag was obtained with resting T cells but not with actively proliferating blasts. Therefore, in all subsequent cytotoxicity assays, effector cells were tested at least 2 wk after the last Ag stimulation.
Preincubation experiments. Effector or ^{51}Cr-labeled target cells were incubated in 96-well microtiter plates in the absence or presence of TUBag4. After a 2- to 4-h incubation at 37°C, plates were spun down for 30" and flicked. Fresh target or effector cells were added on effector or target cell pellets, respectively, mixed, and incubated for 4 h at 37°C in the absence or presence of TUBag4. Supernatants were harvested, counted in a gamma counter, and % specific target lysis was calculated as described previously (24). In some experiments, cells were maintained in suspension during preincubation by gentle pipetting every 15 min.

TNF assays

Supernatants were assayed for TNF using the previously described cytotoxic assay against WEHI164 clone 13 cells (25). Although this cell line is sensitive to human TNF-α and TNF-β, which are both produced upon T cell activation, the fact that WEHI164 cell lysis induced by supernatant from activated T cell clones was almost abrogated by a blocking anti-TNF-α antiserum at 10 μg/ml (Boehringer) indicated that T cells mainly produced TNF-α after activation (data not shown). Each supernatant was tested at two dilutions (1:10 and 1:50) in triplicate. The assay was performed as follows: 50 μl of diluted supernatant was added to 50 μl of actinomycin D-treated (2 μg/ml in PBS, Sigma) WEHI cells (6 × 10^{5} cells/ml) in flat-bottom 96-well plates and incubated for 18 h at 37°C. In each experiment, a reference curve was obtained using serial dilutions of mouse rTNF-α (Boehringer), starting at 100 down to 0.02 pg/ml. After incubation, 50 μl of tetrazolium salts (MTT, 2.5 mg/ml in PBS, Sigma) was added to each well and incubated for 3 h. Formazan crystals were solubilized with 100 μl of lysis buffer (1 vol of N,-N-dimethyl formamide, 2 vol of 30% SDS, adjusted at pH 4.7 with acetic acid), and optical density was read at 570 nm with an ELISA plate reader (Molecular Devices, Les Ulis, France). The TNF content of supernatants was determined by comparing to the standard curve the dilution of supernatant that fell within the linear portion of the curve.

Results

Activation of MHC-unrestricted cytolytic activity of Vγ9Vδ2 T cell clones by TUBag

We and others (16–18) have described nonpeptidic mycobacterial compounds (referred to as TUBag by Constant et al. (17)) that are able to stimulate specifically the proliferation of Vγ9Vδ2 T cells. To investigate in more detail the mode of action of TUBag, we studied their ability to trigger cell lysis mediated by the Vγ9Vδ2 T cell clone G115 (26) in short-term ^{51}Cr release assays. As shown in Table I, TUBag triggered the cytolytic activity of G115 CTL against a broad set of targets but had no effect on target cell lysis mediated by an MHC-restricted αβ CTL clone (BH) (24). TUBag-induced cytolysis was not restricted to targets of a particular tissue or species origin,

Table I.   *Activation of broad killing activity of a Vγ9Vδ2 T cell clone by TUBag*

Species	Target		TUBag-Induced Lysis		LA-Induced Lysis	
	Name	Origin	G115	BH	G115	BH
Human	DAB	BLCL	32	0	80	60
	BL70	BL	21	0	62	32
	BL2/95	BL	37	3	67	32
	RJ225	BL	25	0	33	70
	RAJI	BL	32	0	68	43
	BL30/95	BL	20	0	75	52
	BL30	BL	34	5	95	48
	KGI	TL	16	0	30	24
	THPI	TL	12	0	69	29
	KE6TG	TL	26	0	26	73
	HSB2	TL	21	0	32	71
	MRC5	FBL	39	0	58	50
Murine	DA2	nd	14	6	50	45
	BW5147	Thymoma	12	0	74	65
	A20	BL	22	0	85	62
	SP2/0	Myeloma	9	0	87	52
	P815	Masto	32	0	65	49
	mean$_{(SD)}$		24$_{(9)}$	1$_{(2)}$	62$_{(21)}$	50$_{(15)}$

Cytolytic activity of a Vγ9Vδ2 CTL clone (G115) and an MHC-restricted αβ T cell clone (BH) against ^{51}Cr-labeled target cells was estimated in the absence or presence of the purified mycobacterial nucleotide conjugate TUBag4 (17) or lectin (LA). TUBag4- and LA-induced lysis were calculated according to the following formula: (% specific lysis in the presence of Ag or mitogen) − (% specific lysis in medium alone), estimated at a 20:1 E:T ratio. LA induced efficient lysis of all the target cells tested by the G115 and BH clones, whereas TUBag triggered target lysis by the G115 clone only. BLCL, B lymphoblastoid cell line; BL, B lymphoma; FBL, fibroblast; masto, mastocytoma. nd, not determined.
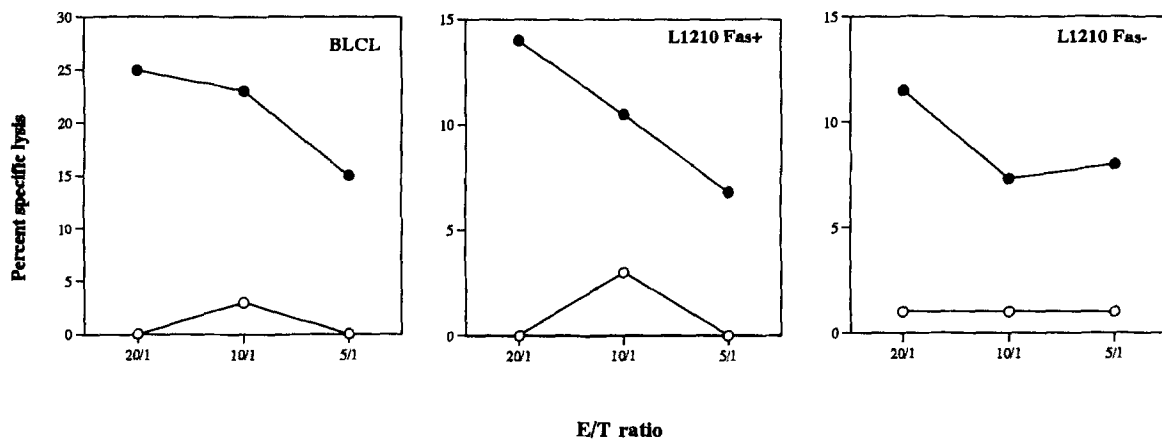

E/T ratio

FIGURE 1.   TUBag activates broad lytic activity of Vγ9Vδ2 cells directed against both Fas$^+$ and Fas$^-$ xenogeneic targets. The cytolytic activity of Vγ9Vδ2 CTL (clone G12) was estimated in a short-term ^{51}Cr-release assay against an allogeneic BLCL (DAB), a Fas$^+$ murine lymphoma (L1210) and its Fas$^-$ variant (L1210-3) at three E:T ratios in the absence (*open symbols*) or presence (*closed symbols*) of ME (1 mg/ml final concentration). Shown are representative data from two independent experiments.

because both hemopoietic and fibroblastic human and murine cells were lysed by clone G115 in the presence of the mycobacterial compound. Moreover, both Fas-positive and Fas-negative xenogeneic targets (27) were killed by G115 clone in the presence of TUBag (Fig. 1), which indicated that this conspicuous killing activity did not result merely from interactions between the broadly distributed Fas molecules and Fas ligands, which are classically expressed by activated CTLs (for review, see Ref. 28).

Induction of Vγ9Vδ2 CTL clone fratricide by TUBag

Strikingly, incubation of Vγ9Vδ2 cells alone in the presence of TUBag led to lysis of the effectors (Fig. 2A). No

effector cell lysis was observed in the presence of TUBag when cells were incubated at 4°C (data not shown), which ruled out a nonspecific toxic effect of the compound. Moreover, G115 cell lysis was abrogated when cells were maintained in suspension during incubation with TUBag, indicating that effector cell lysis resulted from a fratricidal activity (i.e., was dependent on cell-to-cell contact) rather than from a cell suicide (Fig. 2A).

To confirm the relationships between TUBag-induced effector lysis and previously shown induction of Vγ9Vδ2 T cell proliferation, we studied the effect of treatment with phosphatases (alkaline phosphatase and nucleotide pyrophosphatase) on the ability of TUBag to activate Vγ9Vδ2
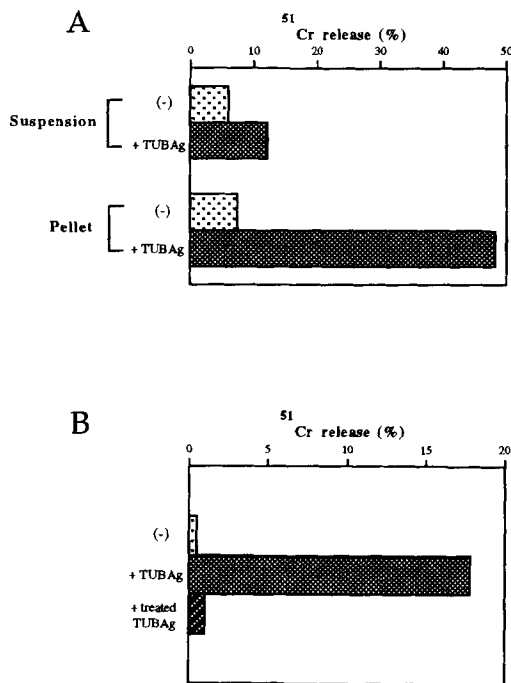
A

^{51}Cr release (%)

B

^{51}Cr release (%)

FIGURE 2. (A) Effect of cell resuspension on TUBag-induced Vγ9Vδ2 effector cell lysis. ^{51}Cr-labeled Vγ9Vδ2 CTL (clone G115) were incubated in the absence or presence of ME (1 mg/ml) at 37°C for 2 h. Cells were either centrifuged at the beginning of the incubation (Pellet) or maintained in suspension by gentle pipetting every 15 min. Results are expressed as % ^{51}Cr-release, calculated by dividing the experimental release by the maximal release (i.e., from cells incubated with detergent). Similar data were obtained in two independent experiments. (B) Dephosphorylation of TUBag abrogates their ability to activate Vγ9Vδ2 T cell fratricide. ^{51}Cr-labeled Vγ9Vδ2 CTL (clone G115) were incubated in the absence (−) or presence of either ME or purified TUBag untreated or treated with a mixture of alkaline phosphatase and nucleotide pyrophosphatase. Shown are representative data obtained with ME of at least five independent experiments.

cells. Consistent with published data (17, 18), dephosphorylation of TUBag abrogated their capacity to stimulate Vγ9Vδ2 T cell proliferation (data not shown) and to induce Vγ9Vδ2 fratricide (Fig. 2B).

In agreement with previous results demonstrating that TUBag induced proliferation of Vγ9Vδ2 T lymphocytes only (17, 18, 29, 30), TUBag triggered fratricide of mycobacteria-reactive CTL clones bearing Vγ9Vδ2 TCR but had no effect on CTL clones bearing αβ TCR or γδ TCR with other VγVδ region combinations (Fig. 3A). Moreover, TUBag-induced G115 CTL lysis was blocked efficiently by mAbs directed against the G115 TCR but neither by irrelevant anti-TCR mAb nor by mAb directed against MHC class I and II framework epitopes (Fig. 3B and data not shown). Of note, none of the TCR-specific mAbs affected lectin-induced fratricide of the effectors (Fig. 3B), which suggested that blockage of TUBag-in-

duced lysis by the mAb was not due to generation of a nonspecific inhibitory signal after TCR cross-linking.

Taken together, the above observations indicated that activation of Vγ9Vδ2 T cell lytic machinery by TUBag was a TCR-dependent process. However, because induction of CTL lysis could be evidenced only through an assay that required cell-to-cell contact, it did not allow us to determine whether activation of Vγ9Vδ2 T cells resulted from direct cognate interactions between soluble TUBag and the γδ TCR or whether it required previous binding of TUBag to the target cells before TCR recognition. To address this issue, we studied induction of TNF production by TUBag as a measure of early T cell activation.

TNF production by T lymphocytes in the presence of TUBag

Incubation of Vγ9Vδ2 T cell clones, but not of αβ T cells, in the presence of TUBag resulted in massive TNF production (generally above 100 pg/1.25 × 10^5 cells after a 3-h incubation) (Fig. 4A and data not shown). Consistent with data drawn from proliferation and cytotoxicity assays, phosphatase-treated TUBag was unable to trigger TNF production by Vγ9Vδ2 cells (Fig. 4A). Of note, TNF secretion by Vγ9Vδ2 T cells was almost abrogated when cells were maintained in suspension in the presence of Ag (Fig. 4B). Hence, this formally demonstrated that efficient induction of TNF production was not induced by soluble TUBag, but required previous binding of these molecules to the cell surface.

Kinetic and preincubation studies

In light of the above results, TUBag-mediated activation of Vγ9Vδ2 T cells was monitored kinetically, to estimate the lag between binding of TUBag to the cell surface and TCR-dependent activation of Vγ9Vδ2 cells. TNF production already was significantly enhanced 20 min after addition of TUBag (10.8 vs 1.6 pg/ml for cells in medium alone; see Fig. 4B). To study early acquisition of lytic activity by Vγ9Vδ2 cells, we performed a cytofluorimetric analysis of propidium iodide (PI) uptake by the effectors, which permitted us to quantify accurately effector fratricide within minutes after addition of the stimulus (Fig. 5A). Although TUBag had no effect on an irrelevant αβ T cell clone, it induced Vγ9Vδ2 T cell clone lysis, which was already detectable 6 min after addition of TUBag (Fig. 5B) and reached a plateau at 60 to 90 min (Fig. 5C).

Target cells lacking the relevant peptidic Ag but carrying appropriate MHC alleles are rendered susceptible to lysis by MHC-peptide-specific αβ CTL after preincubation with Ag before the CML assay. To test whether this also held true for TUBag, effector or target cells were preincubated at 4 or 37°C with TUBag and washed before the cytotoxicity assay, performed with or without TUBag at 37°C. Preincubation of target cells with TUBag followed by a single wash did not render the cells susceptible to
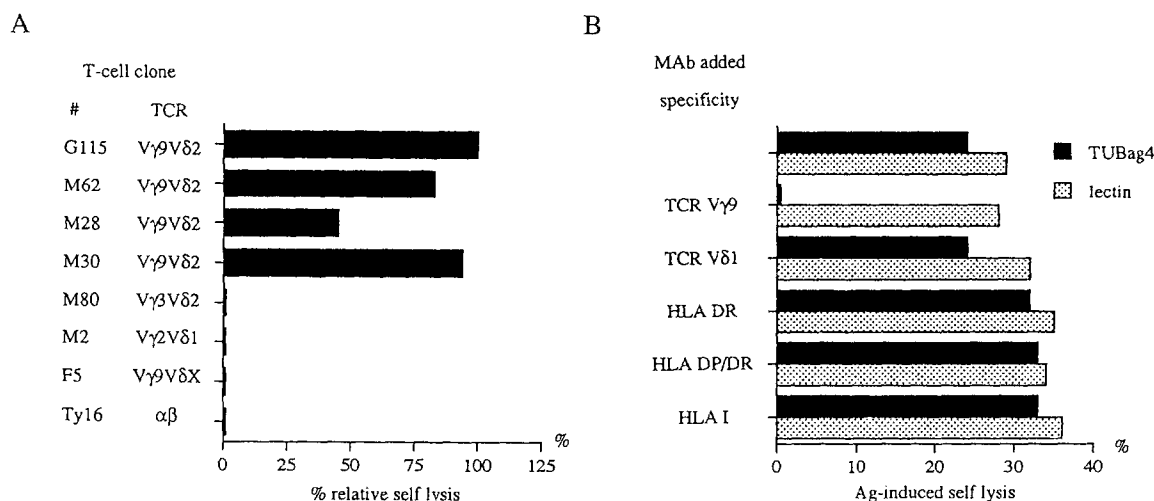
A

B

FIGURE 3. (*A*) TUBag4 induces fratricidal lysis of Vγ9Vδ2 CTL clones only. ^{51}Cr-labeled CTL clones expressing αβ or γδ TCR with various combinations of TCR V regions were incubated for 4 h at 37°C in medium, TUBag4 or leucoagglutinin (LA). To take into account the lytic potential of the CTL clones tested, results were expressed as % relative self-lysis, calculated according to the following formula: [(% lysis in the presence of TUBag4)/(% lysis in the presence of LA)] × 100. Shown are data obtained with T cell clones tested in the same experiment. (*B*) Inhibition of TUBag4-induced fratricidal lysis of a Vγ9Vδ2 T cell clone (G115) by anti-TCR mAbs. ^{51}Cr-labeled G115 cells were incubated for 4 h at 37°C in medium alone or in the presence of saturating amounts of TUBag4, with or without the following mAb (1/100 ascitis): IMMU49 (anti-G115 clonotype), IMMU360 (anti-Vγ9), IMMU389 (anti-Vδ2), IH1 (anti-Vδ1), PL8 (anti-HLA DR), 2D6 (anti-HLA DP/DR), and W6/32 (anti-HLA I). The Vγ9-, Vδ2- and clonotype-specific mAb abrogated TUBag4-induced G115 lysis but had no effect on lectin-induced G115 lysis (see Fig. 1*b* and data not shown). Shown are representative data of three separate experiments.

lysis by Vγ9Vδ2 CTL, even after an overnight incubation (see representative experiment shown in Table II). Preincubation of effector cells with TUBag abrogated their ability to lyse targets, even in the presence of TUBag, probably as a consequence of effector fratricide (Table II). To avoid this TUBag-induced autocytotoxicity, Vγ9Vδ2 cells were maintained in suspension during preincubation with TUBag. Under these conditions, even a 2-h preincubation at 37°C of effectors with saturating amounts of TUBag had no effect on their ability to kill themselves (Fig. 6) or allogeneic targets (data not shown) in the absence or presence of TUBag.

In conclusion, these observations indicated that TUBag-mediated activation of Vγ9Vδ2 cells was very rapid and strictly required the presence of TUBag.

Discussion

Activation of Vγ9Vδ2 cells by mycobacterial antigens thus far has been studied almost exclusively through proliferation assays. These studies have demonstrated clearly the nonpeptidic nature of the activating TUBag mycobacterial compounds (16–18), the lack of MHC restriction of TUBag-mediated activation (30, 31) and the involvement of the TCR in the activation process (16–18, 30, 31). However, because of the lag required between initiation of the proliferation assay and readout, the precise mode of action of these TUBag thus far has remained unclear. In particular, it could not be determined whether these com-

pounds were recognized directly by the Vγ9Vδ2 TCR, either in soluble or membrane-bound form or, alternatively, whether TUBag induced surface determinants recognized in turn by the Vγ9Vδ2 TCR. Moreover, because activation of T cell proliferation classically requires additional accessory signals provided by professional APC (e.g., macrophages or BLCLs), it has not been possible to determine whether TUBag could be "presented" by the T cell responders themselves and/or by other nonprofessional APC of various origins. Clear answers to some of the above issues are provided in the present study, which focused on the analysis of early activation events such as induction of cytotoxicity and TNF production, which classically occurs within minutes or hours after antigenic stimulation.

Our present data indicate that TUBag activated very rapidly and in a highly specific fashion cytotoxicity and TNF production by Vγ9Vδ2 T cells. Significantly, almost any cell (transformed or not, auto-, allo-, or xenogeneic) was killed by Vγ9Vδ2 cells in the presence of TUBag. Several explanations can be put forward to account for this broad killing activity. Because activation of Vγ9Vδ2 cells by TUBag occurred in the absence of any exogenous APC (Fig. 2), it is possible that broad cytotoxicity did not involve recognition of TUBag on the targets but, instead, was the mere consequence of activation of Vγ9Vδ2 cells after self-presentation of TUBag. In this respect, early studies have shown that
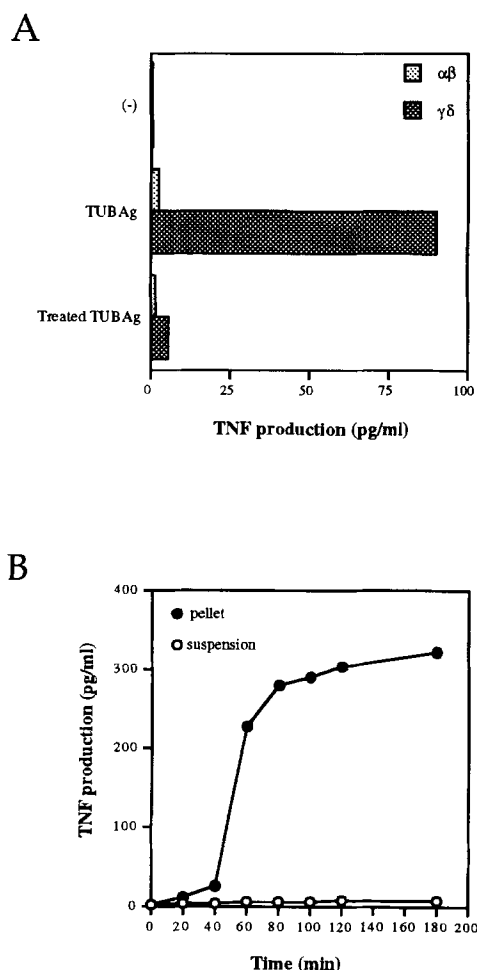
A

B

FIGURE 4. (A) Induction of TNF production by TUBag is specific to Vγ9Vδ2 T cells and is abrogated by phosphatase treatment of TUBag. TNF production by a CD4⁺ αβ and a Vγ9Vδ2 T cell clone (HERP1 and G12, respectively) was estimated after a 3-h incubation in medium alone (−) or in medium supplemented with ME before (TUBag) and after (treated TUBag) treatment with alkaline phosphatase and nucleotide pyrophosphatase. Specificity of the induction process was confirmed in at least five other experiments using 10 different CD4⁺ and CD8⁺ αβ T cell clones and two different Vγ9Vδ2 T cell clones (G12 and G115). Abrogation of TUBag activity by dephosphorylation was confirmed in two distinct experiments. (B) Kinetic analysis of TNF production by Vγ9Vδ2 T cells in the presence of TUBag. Vγ9Vδ2 T cells (clone G12) were incubated in pellet (closed symbols) or in suspension (open symbols) in the presence of ME, and the amounts of secreted TNF were estimated in supernatants recovered at various time points after addition of TUBag. Shown are representative data from two separate experiments.

after specific antigenic stimulation, CTLs could lyse irrelevant targets in their vicinity, presumably in a TCR-independent manner (32). Although this possibility cannot be ruled out formally, it does not fit with the fact that TUBag-induced lysis of exogenous targets was still observed in the absence of detectable self-lysis (our un-

published observations). Therefore, our observations would suggest, rather, that induction of broad killing activity truly resulted from a TCR-dependent recognition of the targets, after binding of TUBag to species-conserved and broadly distributed surface molecules.

At least three distinct mechanisms of cell lysis have been described, and they involve TNF (33), Fas (34, 35), and Perforin (35), respectively. The inability of anti-TNF-α antiserum to block TUBag-induced effector fratricide allowed us to rule out involvement of TNF-α in the lytic process (Fig. 7). Moreover, the fact that Vγ9Vδ2 cells were able to lyse both Fas⁺ and Fas⁻ xenogeneic targets indicated that TUBag-induced broad cytotoxicity was mostly Fas-independent. Accordingly, we found no evidence for induction of cell DNA fragmentation by TUBag (our unpublished results), an apoptotic process that classically follows Fas/Fas-ligand interaction (34, 35). Therefore, although we cannot exclude formally a role of other necrosis-inducing factors (such as lymphotoxin) in the process, it seems more likely that TUBag-induced fratricide is Perforin-based.

How can we reconcile the present observations demonstrating occurrence of Vγ9Vδ2 cell fratricide after TUBag addition and previously shown induction of Vγ9Vδ2 T cell proliferation by the same compounds? In fact, one should stress that although TUBag efficiently activated fratricide of resting Vγ9Vδ2 T cells, it had almost no effect on actively proliferating blasts. Moreover, although induction of effector fratricide was very significant (although never total) when TUBag was added to effector cells alone, it was much weaker when effector cells were cocultured with a large excess of exogenous BLCLs, as was the case for proliferation assays (17).

One might wonder how TUBag activates Vγ9Vδ2 T cells. The need for a cell-to-cell contact to activate effector cell lysis and TNF production clearly indicates that TUBag must be bound to the cell surface before recognition. Whether these compounds have to be adsorbed non-specifically on the target surface to cross-link Vγ9Vδ2 TCR, whether they are recognized together with yet unknown specific receptors or whether they induce surface determinants recognized by the TCR still remains open. Kinetics experiments indicate that if there is an induction of a Vγ9Vδ2 TCR ligand by TUBag, this induction process must be quite rapid because activation of Vγ9Vδ2 cells occurred within minutes after addition of TUBag. Moreover, our inability to detect significant target lysis after removal of TUBag indicates that expression of the induced determinant is very transient. Therefore, the above observations fit rather with the hypothesis of a direct recognition of TUBag by Vγ9Vδ2 TCR, presumably in association with a conserved surface molecule. In this case, the lack of sensitization of target cells to γδ effector lysis reflect merely a low affinity binding of TUBag to its putative receptor(s). Although this second hypothesis still remain unproven, it is clearly conceivable in light of
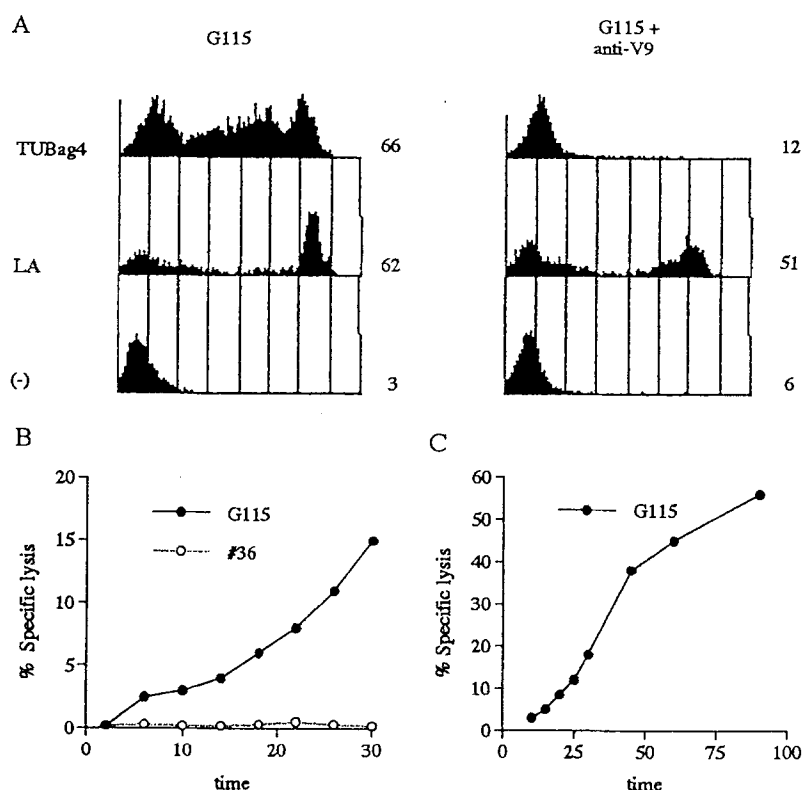
FIGURE 5. (A) CTL clone fratricidal lysis after incubation with TUBag4, as assessed by PI uptake. A Vγ9Vδ2 CTL clone (G115) (26) was incubated for 45 min at 37°C in medium alone (−) or supplemented either with TUBag4 or LA, in the absence (*left*) or presence (*right*) of a Vγ9-specific mAb (IMMU360). Cells were then resuspended in PBS + PI (1 μg/ml, final concentration) and analyzed by flow cytometry. Shown are the red fluorescence histograms (log scale) and the % of PI-positive cells (*right of each histogram*). Note that addition of TUBag4 lead to lysis of G115 cells (*upper left histogram*), which was blocked in the presence of the Vγ9-specific mAb (*upper right histogram*). Similar data were obtained in three independent experiments. (*B*, and *C*) Kinetic analysis of TUBag4-induced self-lysis of a Vγ9Vδ2 CTL clone. Proportions of PI-positive cells (*ordinate*) were studied at various time points (*abscissa*, in min) after addition of TUBag4. In *B*, two effector cells were tested: the Vγ9Vδ2 CTL clone G115 and a polyclonal αβ T cell line (#36). Shown are representative data from three distinct experiments.

Table II. *Effect of effector or target cell preincubation with TUBag on cell susceptibility to lysis by a Vγ9Vδ2 CTL clone*

Cell Preincubated with TUBag	TUBag in CML	% Target Lysis	% Effector Lysis
None	−	5	3
	+	40	47
Target (DAB)	−	4	2
	+	36	47
Effector (G115)	−	13	43
	+	12	55

Cytolytic activity of a Vγ9Vδ2 clone (G115) against ^{51}Cr-labeled DAB BLCL was estimated in the absence or presence of purified TUBag4 (saturating amounts) after preincubation at 37°C with medium or TUBag4 (see *Materials and Methods*). % target lysis was calculated as described previously (24). % effector cell lysis was estimated by trypan blue exclusion (mean of duplicate counts on independent wells).

a recent study demonstrating recognition by some CD4−CD8− αβ T cells of nonpeptidic compounds (i.e., mycophenolic acids) presented by conserved CD1 molecules (36).

Although several issues remain open with respect to the mode of action of TUBag, our present data indicate that
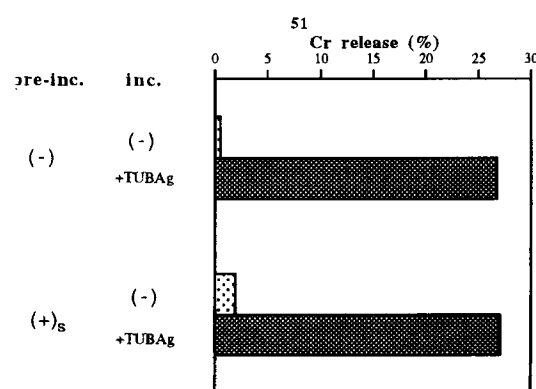
FIGURE 6. Effect of preincubation of Vγ9Vδ2 CTL with TUBag on effector fratricide in the absence or presence of TUBag. ^{51}Cr-labeled Vγ9Vδ2 CTL (clone G115) were incubated in suspension for 2 h at 37°C in medium alone (−) or in medium supplemented with purified TUBag4 (+). Cells were washed once and incubated in the absence or presence of TUBag for 2 h at 37°C before estimation of ^{51}Cr-release. Shown are representative data from three separate experiments.
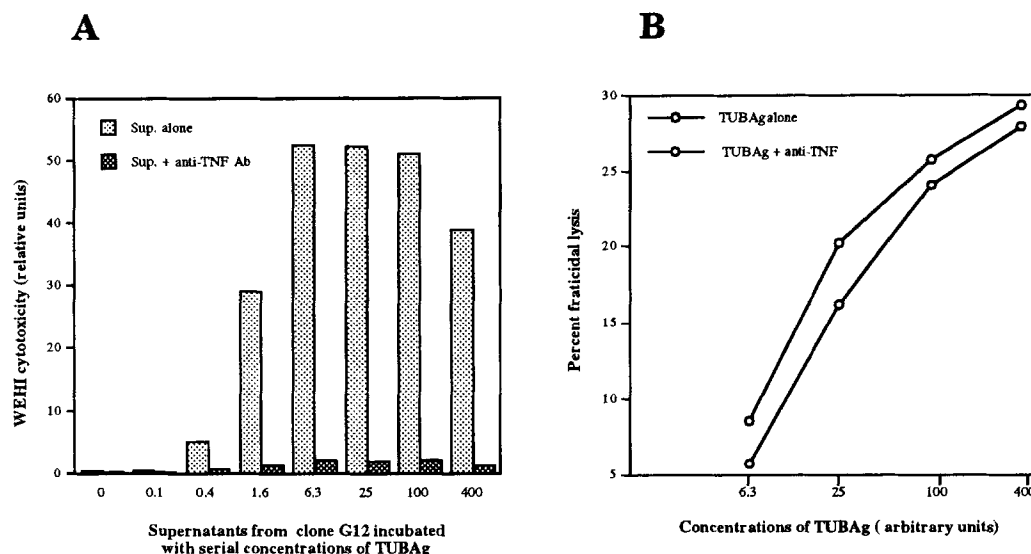
A

B

Supernatants from clone G12 incubated
with serial concentrations of TUBAg

Concentrations of TUBAg (arbitrary units)

FIGURE 7. Effect of an anti-TNF-α antiserum on WEHI164 cytotoxicity and fratricide of a TUBag-activated Vγ9Vδ2 T cell clone. (A) The G12 Vγ9Vδ2 T cell clone was exposed to serial concentrations of TUBag at 37°C for 3 h, and its supernatant was tested for WEHI cytotoxicity in the presence (dark bars) or absence (light bars) of anti-TNF-α antiserum (10 μg/ml). Cytotoxicity is expressed in units relative to cytotoxicity obtained with 100 pg/ml mouse rTNF-α. (B) ^{51}Cr-labeled G12 clone was incubated at 37°C in the absence (open circles) or presence (filled circles) of anti-TNF-α antiserum (10 μg/ml). Effector fratricide was estimated by % of ^{51}Cr released after 3 h of incubation.

these mycobacterial compounds trigger an early and unrestricted cytotoxic response mediated by Vγ9Vδ2 T lymphocytes. Such a powerful activation mechanism might explain the necrotic lesions frequently observed around $\gamma\delta$ T cell infiltrates of mycobacterial infection foci (12–14). These data also lend support for a regulatory role of $\gamma\delta$ T cells, already suggested by previous studies (37–40). Finally, because distribution of Vγ9Vδ2-activating molecules does not seem to be restricted to mycobacteria, these cytotoxic responses to conserved Ags might have important physiopathological implications for other types of bacterial and parasitic infections or autoimmune diseases (19, 30, 41).

Acknowledgments

References

1. Raulet, D. H. 1989. The structure, function and molecular genetics of the $\gamma\delta$ T cell receptor. Annu. Rev. Immunol. 7:175.

2. Haas, W., P. Pereira, and S. Tonegawa. 1993. $\gamma\delta$ T cells. Annu. Rev. Immunol. 11:637.

3. Matis, L. A., R. Cron, and J. A. Bluestone. 1987. Major histocompatibility complex-linked specificity of $\gamma\delta$ receptor-bearing T lymphocytes. Nature 330:262.

4. Bluestone, J. A., R. Q. Cron, M. Cotterman, B. A. Houlden, and L. A. Matis. 1988. Structure and specificity of T cell receptor $\gamma\delta$ on major histocompatibility complex antigen-specific CD3$^+$, CD4$^-$, CD8$^-$ T lymphocytes. J. Exp. Med. 168:1899.

5. Ito, K., L. Van Kaer, M. Bonneville, S. Hsu, D. B. Murphy, and S. Tonegawa. 1990. Recognition of the product of a novel MHC TL region gene (27b) by a mouse γ/δ T cell receptor. Cell 62:549.

6. Ciccone, E., O. Viale, D. Pende, A. Moretta, and L. Moretta. 1989. Specificity of human T lymphocytes expressing a $\gamma\delta$ T cell receptor: recognition of a polymorphic determinant of HLA class I molecules by a $\gamma\delta$ clone. Eur. J. Immunol. 19:1267.

7. Kozbor, D., G. Trinchieri, and D. S. Monos. 1989. Human TCR $\gamma\delta^+$, CD8$^+$ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion. J. Exp. Med. 169:1847.

8. Porcelli, S., M. B. Brenner, J. L. Greenstein, S. P. Balk, C. Terhorst, and P. A. Bleicher. 1989. Recognition of cluster of differentiation 1 antigens by human CD4$^-$CD8$^-$ cytolytic T lymphocytes. Nature 341:447.

9. Mami-Chouaib, F., C. Miossec, P. del Porto, C. Flament, F. Triebel, and T. Hercend. 1990. T-cell target 1 (TCT-1): a novel target molecule for human nonmajor histocompatibility complex-restricted T lymphocytes. J. Exp. Med. 172:1071.

10. Wright, A., J. E. Lee, M. P. Link, S. D. Smith, W. Caroll, R. Levy, C. Clayberger, and A. M. Krensky. 1989. Cytotoxic T lymphocytes specific for self tumor Ig express T cell receptor δ chain. J. Exp. Med. 169:1557.

11. O'Brien, R. L., M. P. Happ, A. Dallas, E. Palmer, R. Kubo, and W. K. Born. 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor $\gamma\delta$ by an antigen derived from Mycobacterium tuberculosis. Cell 57:667.

12. Holoshitz, J., F. Koning, J. E. Coligan, J. De Bruyn, and S. Strober. 1989. Isolation of CD4$^-$ CD8$^-$ mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. Nature 339:226.

13. Haregowoin, A., G. Soman, R. C. Hom, and R. W. Finberg. 1989. Human $\gamma\delta$ T cells respond to mycobacterial heat-shock protein. Nature 340:309.

14. Modlin, R. L., C. Pirmez, F. M. Hofman, V. Torigian, K. Uyemura, T. H. Rea, B. R. Bloom, and M. B. Brenner. 1989. Lymphocytes bearing antigen-specific $\gamma\delta$ T-cell receptors accumulate in human infectious disease lesions. Nature 339:544.

15. Sciammas, R., R. M. Johnson, A. I. Sperling, W. Brady, P. S. Linsley, P. G. Spear, F. W. Fitch, and J. A. Bluestone. 1994. Unique antigen

recognition by a herpesvirus-specific TCR γδ T cell. *J. Immunol. 152:* 5392.

16. Pfeffer, K., B. Schoel, H. Gulle, S. H. E. Kaufmann, and H. Wagner. 1990. Primary responses of human T cells to mycobacteria: a frequent set of γδ T cells are stimulated by protease-resistant ligands. *Eur. J. Immunol. 20:1175.*

17. Constant, P., F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J. J. Fournié. 1994. Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands. *Science 264:267.*

18. Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R. L. Modlin, M. B. Brenner, B. R. Bloom, and C. T. Morita. 1994. Nonpeptide ligands for human γδ T cells. *Proc. Natl. Acad. Sci. USA 91:8175.*

19. Fisch, P., M. Malkovsky, S. Kovats, E. Sturm, E. Braakman, B. S. Klein, S. D. Voss, L. W. Morissey, R. De Mars, W. J. Welch, R. L. H. Bolhuis, and P. M. Sondel. 1990. Recognition by human Vγ9Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science 250:1269.*

20. Kaur, I., S. D. Voss, R. S. Gupta, K. Schell, P. Fisch, and P. M. Sondel. 1993. Human peripheral γδ T cells recognize HSP60 molecules on Daudi Burkitt's lymphoma cells. *J. Immunol. 150:2046.*

21. Davodeau, F., I. Houde, G. Boulot, F. Romagné, A. Necker, N. Canavo, M. A. Peyrat, M. M. Hallet, H. Vié, Y. Jacques, R. Marriuzza, and M. Bonneville. 1993. Secretion of disulfide-linked human T cell receptor γδ heterodimers. *J. Biol. Chem. 268:15455.*

22. Davodeau, F., M. A. Peyrat, I. Houde, M. M. Hallet, H. Vié, and M. Bonneville. 1993. Peripheral selection of antigen receptor junctional features in a major human γδ T cell subset. *Eur. J. Immunol. 23:804.*

23. Davodeau, F., M. A. Peyrat, I. Houde, J. Gaschet, R. Vivien, H. Vié, and M. Bonneville. 1993. Close correlation between Daudi and mycobacterial antigen recognition by human γδ T cells and expression of V9JPC1γ/V2DJCδ-encoded T cell receptors. *J. Immunol. 151: 1214.*

24. Gaschet, J., B. Mahé, N. Milpied, M. C. Devilder, B. Dreno, J. D. Bignon, F. Davodeau, M. M. Hallet, M. Bonneville, and H. Vié. 1993. Specificity of T cells invading the skin during acute graft vs host disease after semi-allogeneic bone marrow transplantation. *J. Clin. Invest. 91:12.*

25. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods 95:99.*

26. Davodeau, F., M. A. Peyrat, I. Houde, M. M. Hallet, G. De Libero, H. Vié, and M. Bonneville. 1993. Surface expression of two distinct functional antigen receptors on human γδ T cells. *Science 260:1800.*

27. Rouvier, E., M. F. Luciani, and P. Goldstein. 1993. Fas involvement in Ca++ independent T cell-mediated cytotoxicity. *J. Exp. Med. 177: 195.*

28. Goldstein, P., M. G. Mattei, C. Foa, and M. F. Luciani. 1994. Molecular mechanisms of T lymphocyte cytotoxicity, with emphasis on the Fas pathway. In *Apoptosis and the Immune Response.* C. D. Gregory, ed. John Wiley & Sons, New York. p. 143.

29. Kabelitz, D., A. Bender, T. Prospero, S. Wesselborg, O. Janssen, and K. Pechold. 1991. The primary response of human γδ positive T cells to *Mycobacterium tuberculosis* is restricted to Vγ9-expressing cells. *J. Exp. Med. 173:1331.*

30. De Libero, G., G. Casorati, C. Giachino, C. Carbonara, N. Migone, P. Matzinger, and A. Lanzavecchia. 1991. Selection by two powerful antigens may account for the presence of the major population of human peripheral γδ T cells. *J. Exp. Med. 173:1311.*

31. Kabelitz, D., A. Bender, S. Schondelmaier, B. Schoel, and S. H. E. Kaufmann. 1990. A large fraction of human peripheral blood TCR γδ positive T cells is activated by *Mycobacterium tuberculosis* but not by its 65 kD heat shock protein. *J. Exp. Med. 171:667.*

32. Lanzavecchia, A. 1986. Is T cell cytotoxicity TCR mediated? *Nature 319:778.*

33. Aggarval, B. B., and J. Vilcek, eds. 1992. *Tumor Necrosis Factor: Structure, Function and Mechanism of Action.* Marcel Dekker, New York, pp. 1–600.

34. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol. Today 16:39.*

35. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Goldstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science 265:528.*

36. Beckman, E. M., S. A. Porcelli, C. T. Morita, S. M. Behar, S. T. Furlong, and M. B. Brenner. 1994. Recognition of a lipid antigen by CD1-restricted αβ+ T cells. *Nature 372:691.*

37. Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. E. Kaufmann. 1993. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. *Nature 365:53.*

38. Kaufmann, S. H. E., C. Blum, and S. Yamamoto. 1993. Crosstalk between αβ T cells and γδ T cells in vivo: activation of αβ T cell responses after γδ T cell modulation with the mAb GL3. *Proc. Natl. Acad. Sci. USA 90:9620.*

39. Fu, Y. X., C. E. Roark, K. Kelly, D. Drevets, P. Campbell, R. O'Brien, and W. Born. 1994. Immune protection and control of inflammatory tissue necrosis by γδ T cells. *J. Immunol. 153:3101.*

40. Lang, F. P., B. H. Pollock, W. J. Riley, N. K. MacLaren, and D. J. Barrett. 1993. The temporal association between γδ T cells and the natural history of insulin-dependent diabetes. *J. Autoimmunity 1:107.*

41. Goerlich, R., G. Häcker, K. Pfeffer, K. Heeg, and H. Wagner. 1991. *Plasmodium falciparum* merozoites primarily stimulate the Vγ9 subset of human γδ T cells. *Eur. J. Immunol. 21:2613.*